PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :
G01N 33/53, 33/537, 33/543
G01N 33/544, 33/546, 33/553
G01N 33/554, 33/555, C12M 1/20
C12M 1/32, 1/34, 1/18

A1

(11) International Publication Number: WO 86/ 07463

(43) International Publication Date: 18 December 1986 (18.12.86)

(21) International Application Number: PCT/US86/00407

(22) International Filing Date: 27 February 1986 (27.02.86)

(31) Priority Application Number: 740,413

(32) Priority Date: 3 June 1985 (03.06.85)

(33) Priority Country: US

(71) Applicant: AMERICAN NATIONAL RED CROSS [US/US]; 400-17th Street, N.W., Washington, DC 20006 (US).

(72) Inventor: NATH, Nrapendra ; 7500 Tarpley Drive, Rockville, MD 20855 (US).

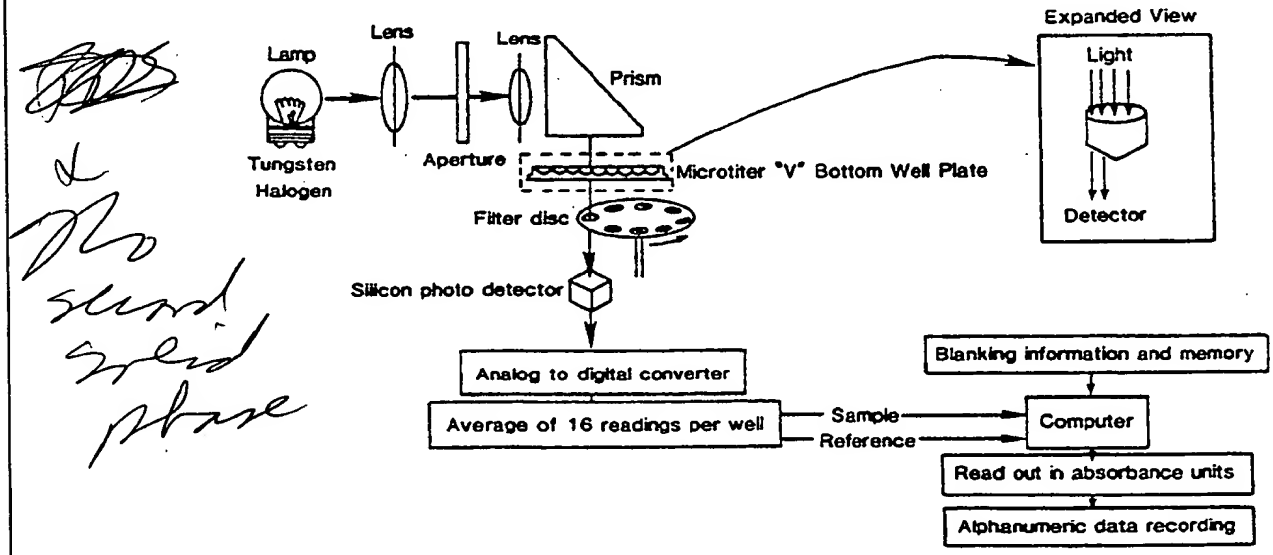
(74) Agents: STERN, Marvin, R. et al.; Holman & Stern, 2401 Fifteenth Street, N.W., Washington, DC 20009 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: MICROTITER - SURFACE - FLOCCULATION ASSAY FOR ANTIGEN OR ANTIBODY SCREENING

SCHEMATIC OF SPECTROPHOTOMETER USED IN MSF ASSAY
(MR-580, DYNATECH LABORATORIES)



(57) Abstract

An objective microtiter-surface-flocculation assay for antigen or antibody screening. The assay employs slanting solid surface coated with an antibody to a protein in a 'V' shaped microtiter well. The assay is readable by instrument means and is automatable fully or partially. A kit containing various components of the assay is also disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BG	Bulgaria
BR	Brazil
CF	Central African Republic
CG	Congo
CH	Switzerland
CM	Cameroon
DE	Germany, Federal Republic of
DK	Denmark
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
HU	Hungary
IT	Italy
JP	Japan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
MC	Monaco
MG	Madagascar
ML	Mali
MR	Mauritania
MW	Malawi
NL	Netherlands
NO	Norway
RO	Romania
SD	Sudan
SE	Sweden
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
US	United States of America

MICROTITER - SURFACE - FLOCCULATION ASSAY FOR ANTIGEN OR ANTIBODY SCREENING.

BACKGROUND OF THE INVENTION

Technical Field

The present invention is related to the detection of antigens or antibodies in a sample. More particularly, the present invention is related to a microtiter - surface-flocculation (MSF) assay for screening the presence of specific antigens or antibodies in a sample of the body fluid.

State of the Art

The most common current test used for screening blood for antibodies, e.g. to syphilis antigen, is by flocculation of charcoal particles coated with cardiolipins from beef heart. The test is done on plastic coated cards and results are determined subjectively, i.e. by visual examination. Clearly, such tests are prone to judgmental errors and are slow due to the necessity of manual manipulation.

Almost all current tests using agglutination/flocculation particles coated with antigen rely on differences in settling patterns of particles in the presence or absence of antibodies. These tests depend for their accuracy and sensitivity upon the training and experience of the person reading the test. Significant variations occur due to the subjectivity of different individuals. The MSF assay of the present invention eliminates subjectivity being machine readable without sacrificing the sensitivity.

Some of the aspects in which the present inventions differs from the currently known assays may be summarized as follows:

Unique features of the present invention:

a) Coating of surface of microtiter plate with antibodies to immunoglubulins resulting in specific differences in the sliding properties of antigen coated particles has not heretofore been used for agglutination/flocculation assays.

b) Coating of solid surfaces with proteins including immunoglobulins is standard laboratory procedure, but it has

not been used to capture specific antibodies that result in changes in flocculation/agglutination pattern specific of antigen-coated particles.

c) The use of microtiter plates to automate the test is not unique, but reading flocculation or agglutination reaction in a coated plate is novel.

d) The use of antibodies to human immunoglobulins to enhance agglutination has been reported, but only in liquid phase and without objective, instrumental reading. The present method is the first to provide a solid phase assay readable by instrument means and being automatable.

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide an objective MSF assay for screening the presence of specific antigens or antibodies in a serum, plasma or a body-fluid sample.

It is a further object of the present invention to provide at least a partially or fully automated MSF assay capable of mechanical reading.

It is yet another object of the present invention to provide a microtiter method for detecting antigen or antibodies in blood which comprises capturing particles coated with specific antigen or antibodies on a slanting surface and determining the amount of flocculation resulting from specific antigen-antibody reaction.

Other objects and advantages of the present invention will become apparent as the detailed description thereof proceeds.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 shows various symbols used in figures 2 and 3 hereof.

Figure 2 shows schematic representaion of various steps for the detection of specific antibodies using MSF assay.

Figure 3 is a schematic representation of various steps for the detection of specific antigens using MSF assay.

Figure 4 is a schematic representation of an embodiment of an automated system for MSF assay.

DETAILED DESCRIPTION OF THE INVENTION

These and other objects of the present invention are achieved by a microtiter-surface-flocculation assay which comprises the steps of (a) coating a slanting solid surface in a microtiter well with antibodies to a protein; (b) adding test sample to the well and incubating the sample for sufficient time at a suitable temperature for binding reaction between the test sample and coated solid surface to be substantially complete; (c) removing unbound sample from step (b); (d) adding to the well particles coated with antibodies or antigens specific for antigens or antibodies, respectively, the presence of which in the sample is to be detected; (e) separating captured antigen-antibody ligand or complex after reaction in step (d) is substantially complete; and (f) reading agglutination reaction by instrument means.

The test sample is not limited to blood or blood products, e.g., serum or plasma, but may be any sample of the body fluid from humans or animals if such body fluid contains or is suspected to contain antigens or antibodies of interest.

The term "substantially complete" as used herein means that the reaction is as complete as can be expected to occur under the conditions within a reasonable time period.

The term "objective" as used herein means that the test result is determined, read or evaluated not by subjective judgment of a person but by instrument means.

Such instruments include a spectrophotometer adopted to read "off-the center" of the microtiter well, a printout or display device to record the reading and the like.

The principle of the MSF assay described herein is that the surface of 'V' wells of microtiter plates is coated with antibodies to a protein, including poly or monoclonal antibodies, preferably human immunoglobulins (IgG, IgM and/or IGA) and the like. These antibodies capture

immunoglobulins from the test sample and when antigen coated charcoal or other suitable paticles are added, these in turn are captured by the specific antibody as shown in Figure 2. Presence of charcoal on the surface of 'V' well interfers with light transmission proportional to the presence of specific antibody in test serum when read in spectrophotometer set to read "off the center" as shown in Figure 4.

Particles such as red blood cells, latex, charcoal magnetic or plastic spheres, fixed stained bacteria and the like can be coated with specific antigen(s) by chemical or physical methods well known in the art. These coated particles when mixed with the body fluid sample, e.g., serum or plasma containing specific antibodies to antigen(s) coated on the particles, cause flocculation or agglutination by forming antigen-antibody linkage or complex with various particles. The difference between negative and positive serum reactions is determined by the pattern and degree of flocculation when particles have settled. The slanting solid surface augments sliding of the flocculated complex to the bottom of the microtiter well and is a unique feature of the present invention.

The present assay can be performed in any suitable plate, utilizing currently FDA licensed reagents and be evaluated automatically or semi-automatically by a machine to provide results in the form of a digital display, printout and the like. The test described herein meets all these criteria. The present assay is particularly suitable in a blood bank type setting where screeing of the samples is required.

The following examples illustrate the preferred embodiments of the present MSF assay.

Example 1-"Test for Syphilis"

100 µl of test serum/plasma are added to a well of coated plate ('V') bottom microtiter plate coated with antibodies to human IgG and IgM heavy chain specific. A set of 3 negatives and 2 positive samples are similarly applied to serve as controls. The plate is covered and incubated at about 37° C for about 60 minutes to allow binding of immunoglobulins in test/control specimens by antibodies coated on the wells. Unbound proteins are removed from the wells by aspiration and washing with phosphate buffered saline (PBS). 100 µl of 1:5 dilution of charcoal particles

coated with syphilis antigen is added to each well except the first well (blank) to which 100 µl of PBS is added. Plates are shaken on rotor (100 rpm) for 10 minutes and then centrifuged at approximatley 1500g for about 1 minute. Reading is taken by employing microplate reader (MR-580, Dynatech) specially modified to read light transmission 'off the center' of the well of microtiter plate as shown in Figure 4. Plates are read using 450 nm as reference and 610 nm as transmission beam in MR-580.

Test Results:

In tests conducted on a panel of positives, 20 samples gave higher light absorbence than negatives. The test values were normalized by determining the net light absorbence i.e., sample-negative control mean or (S-N) value. A sample was considered reactive when S-N was equal or greater than 0.05. The cutoff value may be further adjusted with a larger number of samples.

Example 2: Test for Antibodies to Cytomegalovirus (CMV) in Human Blood

Wells of 'V' bottom plates are coated with anti-human IgG/IgM. After incubating serum or plasma in the well for appropriate time and temperature, unbound serum/plasma proteins are removed by washing with buffer. Latex particles coated with CMV antigen are then added to 'V' wells. After proper incubation, the plate is centrifuged and read for light transmission through the wells. Specimens containing anti-CMV activity block more light than negative specimens. Significant difference between negative and positive samples is obtained.

Example 3: Test for Hepatitis B Surface Antigen (HBsAg):

As shown in Figure 3, 'V' bottom plates are coated with antibodies to HBsAg. HBsAg in test specimen is captured by anti-HBs on 'V' plate and when particles (latex, charcoal or red cells) coated with anti-HBs are added, these bind to HBsAG already captured by anti-HBs coated on the wells of the plate. After centrifugation plates are read for light absorbance as described supra.

Similar tests may be utilized for antibodies to Brucellosis. Of course, the present MSF assay can be employed for antigen as well as antibodies in any system where particle agglutination occurs. Clearly, the MSF assay of the present invention is inherently convenient, efficient and superior to currently employed manual subjective assays.

An MSF kit and an MSF apparatus are two other embodiments of the present invention. The components of the kit and/or the appartus comprise microtiter plate having a plurality of 'V' shaped wells; solid surface coated with an antibody to a protein, preferably to IgG/IgM/IgA; container(s) containing specific antigen or antibody coated particles; container containing a suitable buffer or washing medium, e.g., PBS; a microtiter reader assembly, preferably with a printout or display device; instructions for carring out the assay and other accessories, e.g., micropipette, and the like commonly included in such kits or devices.

An at least partially automated device for microtiter surface flocculation assay for detecting the presence of a specific antibody in a sample is now described. The device comprises a 'V' shaped container transparent to light in the visible spectrum for receiving said sample; a solid surface coated with an antibody to a

protein, said surface being slantingly disposed in said container; means for introducing in said container a predetermined quantity of particles coated with an antigen specific to the antibody presence of which is to be detected; means for separating from the sample in said container antigen-antibody complex formed as a result of reaction between said antigen coated particles and the antibody in said sample; means for detecting the presence of said complex in said container and means for recording the result thereof. The recording means may be any suitable assembly, preferably a printout or a display device.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

IN THE CLAIMS:

1. A microtiter-surface-flocculation (MSF) assay for detecting the presence of an antibody, comprising the steps of:

a) coating a slanting solid surface in a microtiter well with poly- or mono-clonal antibodies to a protein;

b) adding test sample to the well and incubating the sample for sufficient time at a suitable temperature for binding reaction between the test sample and coated solid surface to be substantially complete;

c) removing unbound sample from step (b);

d) adding to the well particles coated with antigens specific for antibodies the presence of which in the sample is to be detected;

e) separating captured antigen-antibody ligand or complex after reaction in step (d) is substantially complete; and

f) reading agglutination reaction by instrument means.

2. The assay of claim 1 wherein said microtiter well is a 'V' shaped well.

3. The assay of claim 2 wherein said protein is selected from the group consisting of immunoglobulin IgG, IgM and IgA.

4. The assay of claim 3 wherein said test sample is a body fluid.

5. The assay of claim 4 wherein said body fluid is a blood sample.

6. The assay of claim 5 wherein said blood sample is plasma or serum.

7. The assay of claim 6 wherein said particle is selected from the group consisting of charcoal, latex and red blood cells, magnetic spheres, plastic spheres and fixed stained bacteria.

8. A method of screening blood samples in a blood bank setting for the presence of specific antibodies, comprising testing said samples by the assay of claim 1.

9. A kit for detecting the presence of antigen or antibodies in a sample comprising: a microtiter plate with 'V' shaped wells; a solid surface coated with antibodies against a protein, said surface being slantingly disposed in said well; a container containing particles coated with a specific antigen or antibody; and instruction for using the kit.

10. An at least partially automated device for microtiter surface flocculation assay for detecting the presence of a specific antigen or antibody in a sample, comprising: a 'V' shaped container transparent to light in the visible spectrum for receiving said sample; a solid surface coated with an antibody to a protein, said surface being slantingly disposed in said container; means for introducing in said container a predetermined quantity of particles coated with an antigen specific to the antibody the presence of which is to be detected; means for separating from the sample in said container antigen-antibody complex formed as a result of reaction between said antigen or antibody coated particles and antigen or antibody in said sample; means for detecting the presence of said complex in said container and means for recording the result thereof.

11. The device of claim 10 having a plurality of said container.

12. The device of claim 11 wherein said detecting means is a spectrophotometer.

13. The device of claim 12 wherein said recording means is a printout or display assembly.

14. A microtiter-surface-flocculation (MSF) assay for detecting the presence of an antigen, comprising the steps of:

a) coating a slanting solid suface in a microtiter well with poly- or mono-clonal antibodies to a protein;

b) adding test sample to the well and incubating the sample for sufficient time at a suitable temperature for binding reaction between the test sample and coated solid surface to be substantially complete;

c) removing unbound sample from step (b);

d) adding to the well particles coated with antibodies specific for antigens the presence of which in the sample is to be detected;

e) separating captured antigen-antibody ligand or complex after reaction in step (d) is substantially complete; and

f) reading agglutination reaction by instrument means.

15. The assay of claim 14 wherein said microtiter well is a 'V' shaped well.

16. The assay of claim 15 wherein said protein is selected from the group consisting of immunoglobulin IgG, IgM and IgA.

17. The assay of claim 16 wherein said test sample is a body fluid.

18. The assay of claim 17 wherein said body fluid is a blood sample.

19. The assay of claim 18 wherein said blood sample is plasma or serum.

20. The assay of claim 19 wherein said particle is selected from the group consisting of charcoal, latex, red

blood cells, magnetic spheres, plastic spheres and fixed stained bacteria.

21. A method of screening blood samples in a blood bank setting for the presence of specific antigens comprising testing said samples by the assay of claim 14.

FIG. 1

Symbols used

96-well microtiter plate, 'V' bottom.

Particles (charcoal, RBcs, latex bacteria or others) coated with target antigen.

Antibodies to heavy chains of human IgG and for IgM. Antibodies could be polyclonal or monoclonal.

Antibodies with specific activity against HBsAg (prepared in animals or human).

Antibodies in test sera with specific activity to target antigen.

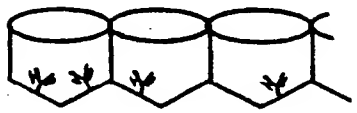
HBsAg particles in blood.

Antibodies in test sera with no specific affinity for the target antigen.

Particles coated with antibody to HBsAg.

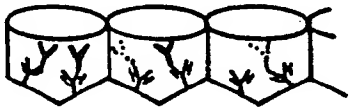
FIG. 2

Detection of specifc antibodies using MSF assay

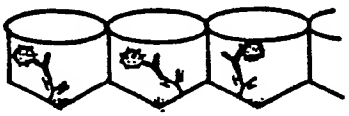


Step #1 Microtiter plate coated with antibodies to human IgG and/or IgM.

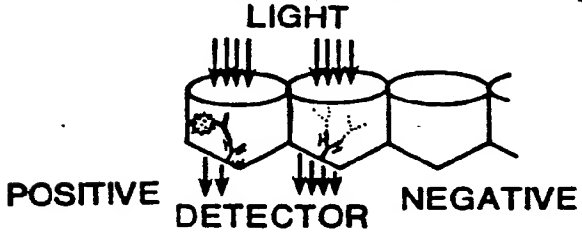
Step #2 Test sera added and incubated. IgG/IgM captured by antibodies coated on the surface of the 'V' bottom well.



Step #3 Wells are washed to remove uncaptured IgG/IgM. A proportion of molecules captured feom test serum will have specific activity to target antigen.



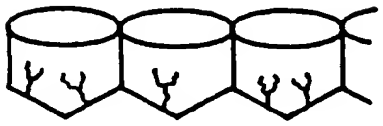
Step #4 Target antigen coated particles added, incubated and centrifuged.



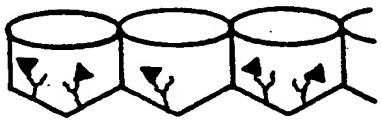
Step #5 Read in spectrophotometer for light absorbed by particles on the wall of the wells of microtiter plate.

FIG. 3

Detection of specific antigen using MSF assay

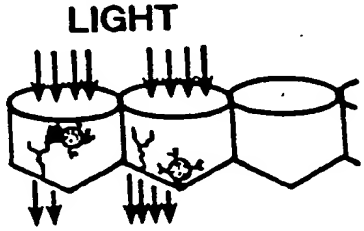


Step #1 Anti-HBs coated on microtiter plate 'V' bottom. Anti-HBs could be polyclonal or monoclonal.



Step #2 Add test serum, incubate, wash. HBsAg in test sera captured by anti-HBs coated on plate.

Step #3 Add anti-HBs coated particles (red cells, latex or charcol particles) incubate. centrifuge.



Step #4 Read for light absorbance in spectrophotometer.

SCHEMATIC OF SPECTROPHOTOMETER USED IN MSF ASSAY
(MR-580, DYNATECH LABORATORIES)

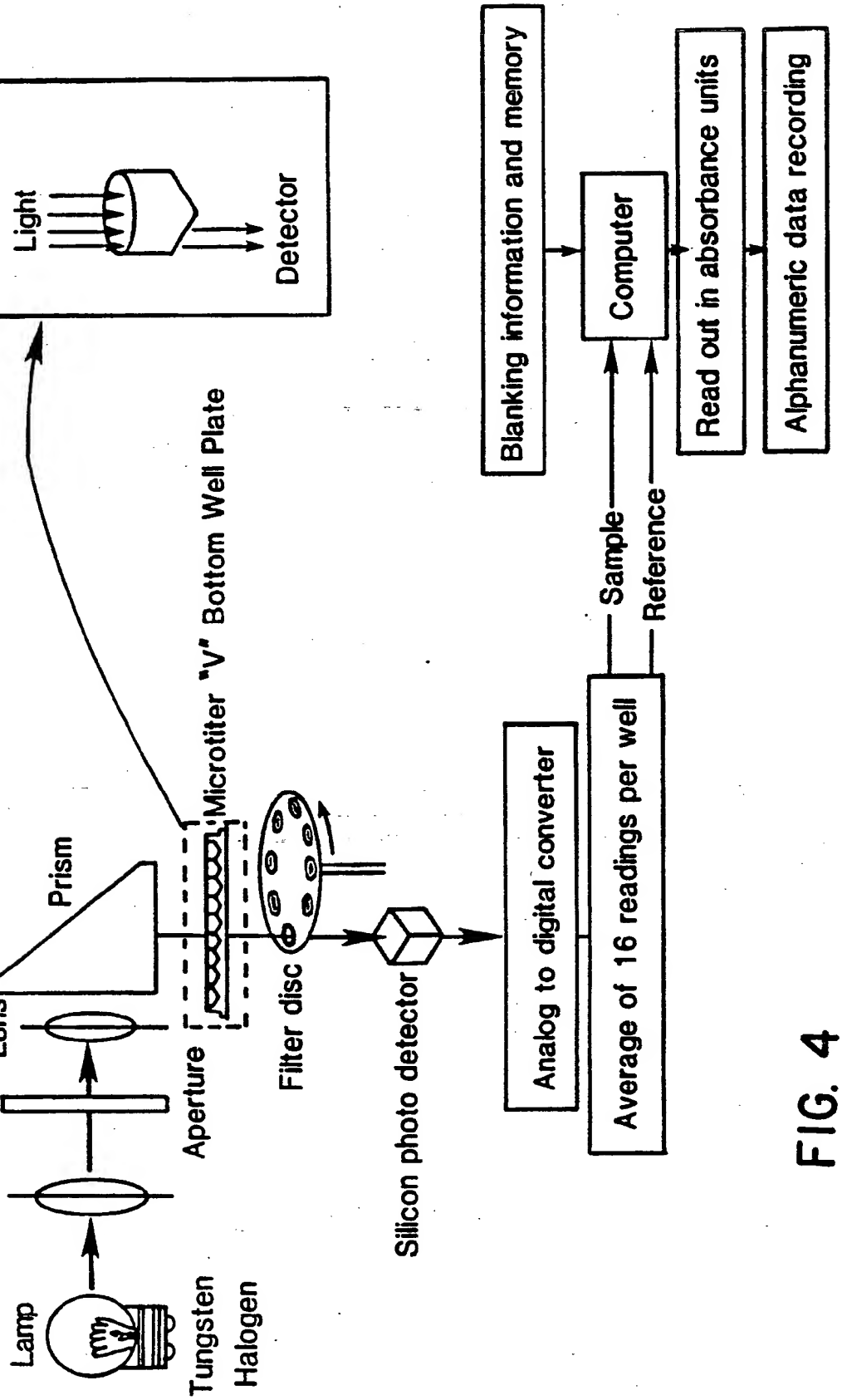


FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/00407

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S.: 436/513,518,519,520,526,528,533,534,538,808,809; See Attachment)
INT. CL.[4]: G01N 33/53,537,543,544,546,553,554,555; See Attachment

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	436/513,518,519,520,526,528,533,534,538,548,808,809 435/291,293,300,301,810 422/73

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	A. Voller et al. (editors), "Immunoassays for the 80s", published 1981, by University Park Press (Baltimore), see pages 29-31 and 354-355, especially page 29.	1-21
Y	A. Bradburne et al., "A solid-Phase System (SPACE) for the Detection and Quantification of Rotavirus in Faeces", J. Gen. Virol., Volume 44, published 1979, see pages 615-623, especially pages 617 and 619.	1-21

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
09 May 1986	15 MAY 1986
International Searching Authority [1]	**Signature of Authorized Officer [10]**
ISA/US	Randall E. Deck

Form PCT/ISA/210 (second sheet) (October 1981)

ATTACHMENT

I. CLASSIFICATION OF SUBJECT MATTER (CONTINUED):

U.S.: 435/291,293,300,301,810
422/73
436/548

INT. CL.[4]
C12M 1/20,1/32,1/34,1/18

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
A	US,A, 4,486,530 (David et al.) 4 December 1984, see column 5, lines 59-63, column 8, lines 37-50 and column 10, lines 51-62.	1-21
Y	US,A, 4,290,997 (Suovaniemi) 22 September 1981, see column 4, lines 4-9 and column 5, lines 41-57.	2,9,10,15
Y	US,A, 4,273,756 (Ling et al.) 16 June 1981, see column 2, lines 43-58.	1-10,16
Y	US,A, 4,347,311 (SCHMITZ) 31 August 1982, see entire document	1-10,16
Y	WO,A1, 82-03462 (Biospecia Limited) 14 October 1982, see page 6.	10-13
Y	DE,A, 3,246,873 (Olympus Optical KK) 07 July 1983, see Figure 1.	10-13
A	WO,A1, 82-00203 (Institut Pasteur) 21 January 1982.	1-21

FURTHER INF RMATI N C NTINUED FR M THE SECOND SHEET

Y	JP,A,	56-151357 (Hitachi Seisakusho K.K.) 24 November 1981, see Figure 1	1-21
A	US,A,	4,444,879 (Foster et al) 24 April 1984, see column 6, lines 49-51 and column 15, lines 12-17.	1-21

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ....., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.